



Atty. Dkt. No. 355908-1300

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Pavel HAMET et al.

Title: PRE-CONDITIONING CELLS
AGAINST CELL DEATH

Appl. No.: 09/480,260

Filing Date: January 11, 2000

Examiner: M. Meller

Art Unit: 1654

DECLARATION UNDER 37 C.F.R. §1.132

I, **Marina A. Lynch, Ph.D.**, hereby declare that:

1. I am Lecturer and Associate Professor at Trinity College, Dublin, Ireland. I have authored or co-authored over 100 published scientific papers in the field of synaptic plasticity over the past 20 years. A copy of my Curriculum Vitae is attached hereto as **Exhibit A**.
2. I am a paid consultant to Vasogen Inc. (Mississauga, Canada) and Vasogen Ireland Ltd. (Shannon, Co. Clare, Ireland) (collectively, "Vasogen"). I have conducted studies funded by Vasogen in the area of assessment of the effects of certain of Vasogen's therapeutic compositions on synaptic activity in rat hippocampus.
3. I am a co-author of the research paper entitled "Attenuation of LPS-Induced Changes in Synaptic Activity in Rat Hippocampus by Vasogen's Immune Modulation Therapy," by Y. Nolan, A. Minogue, E. Vereker, A. E. Bolton, V.A. Campbell and M.A. Lynch, published in the journal *Neuroimmunomodulation*, volume 10, pages 40-46 (2002-2003), (hereinafter, the "Nolan Paper"). A copy of this paper is attached hereto as **Exhibit B**.
4. I have reviewed the subject U.S. Patent Application Serial Number 09/480,260 (hereinafter, the "Hamet Application") and the U.S. Patent Examiner's remarks concerning enablement of claims directed to methods of alleviating or protecting against the symptoms of a medical disorder involving accelerated rates of apoptosis.
5. The Hamet Application describes a method of reducing a mammalian subject's susceptibility to a condition characterized by apoptotic death of cells, by administering to the

subject an aliquot of blood that has been subjected to one or more of certain stressor condition(s) ("IMT Treatment"). The results reported in the Hamet Application indicate that animals who receive such treatment exhibit lower levels of apoptotic cell death in areas subjected to apoptosis-inducing events.

6. I have studied the phenomenon of apoptotic cell death in an experimental model of brain cell function, the electrophysiological measurement of long term potentiation (LTP) in the hippocampus of the anaesthetized rat. This preparation is exquisitely sensitive to systemic administration of lipopolysaccharide (LPS), an inducer of pro-inflammatory cytokines. As shown in the Nolan Paper, peripheral administration of LPS resulted in increased levels of the inflammatory cytokine IL-1 (C.f., Figure 2, page 43), increased apoptotic cell death, as measured by TUNEL staining (C.f., Figure 5, page 45), as well as decreased synaptic function, as evidenced by reduced LTP activity in LPS-treated animals, as compared to control animals (Figure 1, page 4).

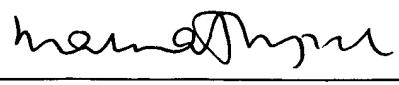
7. In experiments reported in the Nolan Paper, my co-authors and I demonstrated that pretreatment of rats with Vasogen's IMT Treatment prevented most if not all of the above negative effects. Specifically, the IMT Treatment inhibited LPS-induced apoptotic changes in brain hippocampal cells (C.f., Figure 5, page 45). Furthermore, the IMT Treatment is a blood stressor treatment that falls within the description of the treatment modality claimed in the Hamet Application.

8. On the basis of our studies, we concluded that "...pretreatment with the [IMT Treatment] confers a protective effect ... by preventing LPS-induced impairment of synaptic function and the resultant detrimental effects in the hippocampus of the rat."(page 46, last paragraph).

9. We therefore showed that Vasogen's IMT Treatment, which is the treatment claimed in the Hamet Application, confers a protective effect on susceptible neuronal cells.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at TCD Dublin, Ireland, this 26 day of August, 2004.



Marina A. Lynch

CURRICULUM VITAE: Marina A Lynch

Name: Marina Annetta LYNCH
Date of Birth: 10th March, 1953
Address: Department of Physiology, Trinity College, Dublin 2
Qualifications: B.Sc. (NUI), MSc. (NUI), PhD (Dubl)

Positions Held

1999-Present	Associate Professor of Physiology, University of Dublin
1992-1998	Lecturer, Department of Physiology, University of Dublin
1988-1992	Research Scientist, National Institute for Medical Research, London
1983-1988	Postdoctoral Fellow, National Institute for Medical Research, London
1981-1983	Postdoctoral Fellow, King's College, London

Institutional Responsibilities include

University of Dublin Academic Affairs Committee; Director of Postgraduate Studies Trinity College Institute of Neuroscience; University of Dublin Life Sciences Strategic Planning Group; University of Dublin Disciplinary Panel

External Responsibilities include

Member: Commission to advise Government on Assisted Human Reproduction; Health Research Board Neuroscience Grants Committee

Reviewer: Wellcome Trust, Science Foundation of Ireland (Chair of Neuroscience panel 2004), Health Research Board, and various learned journals including J Neuroscience, Neuroscience, European J Neuroscience, Neuropharmacology, J Neurochemistry

Awards and Distinctions include

MRC Postdoctoral Fellowship (1981-1988)

Honorary Lecturer, Royal Free Hospital School of Medicine, London (1990-1992)

Elected to Fellowship, Trinity College Dublin (1997)

Plenary lectureships include (most recent).

Hippocampal Research Conference, Grand Cayman (2000), Society for Neurosciences, New Orleans (2000), Neurobiology of interleukin-1 receptors (EU symposium), Biarritz (2000), European Society of Neurochemistry, Perugia (2001), Portuguese Society of Neuroscience, Peniche (2001), American Aging Association, San Diego (2002), IBRO World Congress of Neuroscience, Prague (2003), PsychoNeuroimmunology Research Society, Titisee Germany (2004), Federation of European Neurosciences, Lisbon (2004). Currently 5 invitations to participate in international conferences in 2005

Invited chapters and reviews (most recent)

Progress in Neurobiology (1998), Reviews in Neurosciences (1998), Molecular Psychiatry (1998), Vitamins and Hormones (2000), Nutritional Neurosciences (2000), Diet – Brain connections: Impact on memory, mood, ageing and disease (2001), Physiological Reviews (2003)

Publications

>120 peer-reviewed research papers, 20 invited reviews and book chapters, 150 conference proceedings

PEER-REVIEWED PAPERS

1. Lynch, M., Kenny, M. and Leonard, B.E. (1977) Changes in the catecholamine content in 4 regions of the rat brain following acute amphetamine treatment: antagonistic effect of chlorpromazine. I.C.R.S. Med. Sci., 5, 561.
2. Lynch, M., Kenny, M. and Leonard, B.E. (1977) Changes in behaviour in rats in the "open field" apparatus after chronic amphetamine administration: acute effects of 3 antidepressants. I.C.R.S. Med. Sci., 5, 571.
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12. Lynch, M. and Littleton, J.M. (1983) Possible association of alcohol tolerance with increased synaptic Ca^{2+} sensitivity. Nature, 303, 175-177.
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14. Lynch, M., Andrews, J.F. and Moore, R.E. (1985) Low doses of T_3 induce a rapid metabolic response in young lambs. Horm. Metabol. Res., 17, 63-66.
15. Lynch, M., Bruton, J.D., Andrews, J.F. and Moore, R.E. (1985) The rapid metabolic response of young lambs to low doses of T_3 : interaction with rT_3 . J. Therm. Biol., 10, 71-77.
16. Lynch, M., Samuel, D. and Littleton, J.M. (1985) Altered characteristics of $[^3\text{H}]$ dopamine

release from superfused slices of corpus striatum obtained from rats receiving ethanol *in vivo*. *Neuropharmacology*, 24, 479-485.

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23. Lynch, M. and Bliss, T.V.P. (1986) On the mechanism of enhanced release of ^{14}C glutamate in hippocampal long-term potentiation. *Brain Res.*, 369, 495-509.
24. Lynch, M., Archer, E.R. and Littleton, J.M. (1986) Increased sensitivity of transmitter release to calcium in ethanol tolerance. *Biochém. Pharmacol.*, 3, 1207-1209.
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Attenuation of LPS-Induced Changes in Synaptic Activity in Rat Hippocampus by Vasogen's Immune Modulation Therapy

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Key Words

Long-term potentiation · Hippocampus · IL-10 · IL-1 β · Lipopolysaccharide · Vasogen's IMT

Abstract

Systemic injection of lipopolysaccharide (LPS) blocks the expression of long-term potentiation in the hippocampus of the rat. This is coupled with increased IL-1 β concentration and c-Jun NH₂-terminal kinase activity, as well as an increase in the number of cells displaying apoptotic characteristics in the hippocampus. Vasogen's Immune Modulation Therapy (IMT) is a procedure involving intramuscular administration of syngeneic blood which has been exposed *ex vivo* to elevated temperature, oxidation and ultraviolet light. We report that Vasogen's IMT significantly abrogates these LPS-induced effects with a concomitant increase in the concentration of the anti-inflammatory cytokine IL-10. These data suggest that Vasogen's IMT may play a protective role against the deleterious effects of immune insults in the brain.

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Introduction

Systemic administration of lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, provokes activation of the immune system by inducing an increase in pro-inflammatory cytokines such as IL-1 β . As well as its effects on the peripheral immune system, LPS is now known to be responsible for stimulating changes in the central nervous system, affecting processes such as thermoregulation, sleep and appetite [1]. One example of a neuronal deficit induced by LPS and IL-1 β is the impairment of long-term potentiation (LTP) in the hippocampus [2, 3]. LTP is a form of synaptic plasticity and has been proposed as a biological substrate for learning and memory [4]. The inhibitory effects of both IL-1 β and LPS on LTP have been linked with an increase in activity of the stress-activated protein kinase c-Jun NH₂-terminal kinase (JNK) [2]. Activation of JNK has been identified as instrumental in bringing about cell function deterioration and, ultimately, cell death [5, 6].

IL-10 is one of a number of cytokines secreted by the T helper 2 (Th2) subclass of lymphocytes and is known for its anti-inflammatory effects. Anti-inflammatory cytokines, such as IL-10, have been reported to prevent IL-1 β -induced changes [7, 8], thus inhibiting pro-inflammatory responses. It has been demonstrated that Vasogen's Immune Modulation Therapy (IMT), which involves intramuscular administration of syngeneic blood following *ex vivo* treatment with elevated temperature, oxidation

and ultraviolet light [9], appears to mediate a Th1 to Th2 immunodeviation in patients suffering from scleroderma, a Th1-mediated disease [10]. There is evidence that Vasogen's IMT suppresses contact hypersensitivity [11] and reduces the progression of atherosclerosis in low-density lipoprotein receptor-deficient mice [12], suggesting a possible anti-inflammatory activity. The proposed role of Vasogen's IMT in interfering with pro-inflammatory responses prompted an investigation into its effect on LPS-induced neuroinflammation. In this study we assessed changes in LTP, IL-1 β concentration, JNK activity and evaluated TUNEL staining for fragmented DNA, a characteristic of apoptotic cells.

Materials and Methods

Animals and Treatment Protocol

Male Wistar rats (300–350 g; BioResources Unit, Trinity College Dublin, Ireland) were used in these experiments. Animals were housed in groups of 4 under a 12-hour light/dark schedule with free access to food and water. Ambient temperature was controlled between 22 and 23°C and rats were maintained under veterinary supervision.

Whole blood (11 ml) was obtained from donor rats by cardiac puncture and added to 2.2 ml of 3.13% sodium citrate solution, of which 1.2 ml was removed and used for sham treatment. For Vasogen's IMT, the remaining 12 ml of anticoagulated blood were transferred to a single-use blood container (VC7002, Vasogen Inc., Toronto, Canada) and exposed to a combination of controlled physiochemical stress factors in a medical device (VC7001, Vasogen Inc.). The medical device executed an automated procedure during which the temperature of the blood was first raised (to a nominal temperature of 42.5°C) over a period of 6–8 min. A gas mixture of ozone in medical oxygen (nominal concentration 14.5 µg/ml) was then applied to the blood (nominal flow rate 240 ml/min) for 3 min. During this time the blood was exposed to UVC light (maximum emission spectrum at 254 nm). Finally, the treated blood was allowed to settle for at least 7 min prior to removal from the blood container. Rats were treated by intramuscular injection of 150 µl of processed blood or untreated blood (sham treatment). Injections were administered 14 days, 13 days and 1 day before LPS or saline challenge. Rats were divided into 4 treatment groups, which will be referred to as sham-saline, sham-LPS, IMT-saline, IMT-LPS.

Induction of LTP in Perforant Path-Granule Cell Synapses *in vivo*

LTP was induced as described previously [13]. Rats were anaesthetised by an intraperitoneal urethane injection (1.5 g/kg), subsequently received either LPS (100 µg/kg) or saline intraperitoneally and were monitored for 3 h. Rats were then placed in a head holder in a stereotaxic frame. A window of skull was removed to allow placement of recording and stimulating electrodes in the molecular layer of the dentate gyrus (2.5 mm lateral and 3.9 mm posterior to bregma) and perforant path (angular bundle, 4.4 mm lateral to lambda), respectively. The depth of the electrodes was adjusted to obtain maximal responses in the cell body region and, after an initial period to

allow baseline responses to stabilise, test shocks were delivered to the perforant path at the rate of 1/30 s. Responses were recorded for 10 min prior to and 40 min following tetanic stimulation (3 trains of stimuli, 250 Hz for 200 ms; intertrain interval 30 s). At the end of the electrophysiological recording period, rats were killed by decapitation, the hippocampus was removed, dissected on ice and cross-chopped into slices (350 µm × 350 µm), using a McIlwain tissue chopper. The time needed to prepare slices from the time of death was 2.5–3.5 min. All samples were frozen separately in 1 ml Krebs solution (composition, in mM: NaCl 136, KCl 2.54, KH₂PO₄ 1.18, MgSO₄·7 H₂O 1.18, NaHCO₃ 16, glucose 10, CaCl₂ 1.13) containing 10% dimethylsulphoxide [14]. For analysis, thawed slices of tissue were rinsed 3 times in fresh ice-cold Krebs solution and homogenized in ice-cold Krebs solution.

Analysis of IL-1 β Concentration

IL-1 β concentration in hippocampal homogenate was analysed by ELISA (R & D Systems, UK). Antibody-coated (100 µl; 1.0 µg/ml final concentration, diluted in phosphate buffered saline (PBS), pH 7.3; goat anti-rat IL-1 β antibody), 96-well plates were incubated overnight at room temperature, washed several times with PBS containing 0.05% Tween 20 and blocked for 1 h at room temperature with 300 µl blocking buffer (PBS, pH 7.3, containing 5% sucrose, 1% bovine serum albumin (BSA), and 0.05% NaN₃). After several washes, plates were incubated with IL-1 β standards (100 µl; 0–1,000 pg/ml in PBS containing 1% BSA) or samples (homogenised in Krebs solution containing 2 mM CaCl₂) for 2 h at room temperature. Samples were incubated with secondary antibody (100 µl; final concentration 350 ng/ml in PBS containing 1% BSA and 2% normal goat serum; biotinylated goat anti-rat IL-1 β antibody) for 2 h at room temperature, washed and incubated in detection agent (100 µl; horseradish peroxidase conjugated streptavidin: 1:200 dilution in PBS containing 1% BSA) for 20 min at room temperature. Substrate solution (100 µl; 1:1 mixture of H₂O₂ and tetramethylbenzidine) was added, samples were incubated at room temperature in the dark for 1 h, the reaction was stopped using 50 µl 1M H₂SO₄. Absorbance was read at 450 nm, values were corrected for protein [15] and expressed as pg/mg protein.

Analysis of IL-10 Concentration

A commercially available ELISA (Biosource International Inc., USA) was used to analyse IL-10 concentration in the hippocampus. Samples were homogenised in Iscove's culture medium containing 5% fetal bovine serum and a cocktail of enzyme inhibitors (100 mM amino-n-caproic acid; 10 mM Na₂EDTA; 5 mM benzamidine HCl; 0.2 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at 10,000 rpm at 4°C for 10 min, the supernatant was removed and analysed for IL-10 using ELISA. Optical densities were determined at 450/630 nm dual wavelength mode using a multi-well plate reader, values were corrected for protein [15] and expressed as pg/mg protein.

Analysis of JNK Activity

The activity of JNK was analysed in homogenate prepared from frozen hippocampal slices. In a separate experiment, activity of the kinase was assessed in freshly prepared hippocampal synaptosomes obtained from untreated rats. These samples had been pre-treated for 20 min in the absence and presence of IL-1 β (1 ng/ml) and vasoactive intestinal peptide (VIP; 1 µM), a proven JNK inhibitor [16]. In all experiments, samples were analysed for protein [15], and diluted to

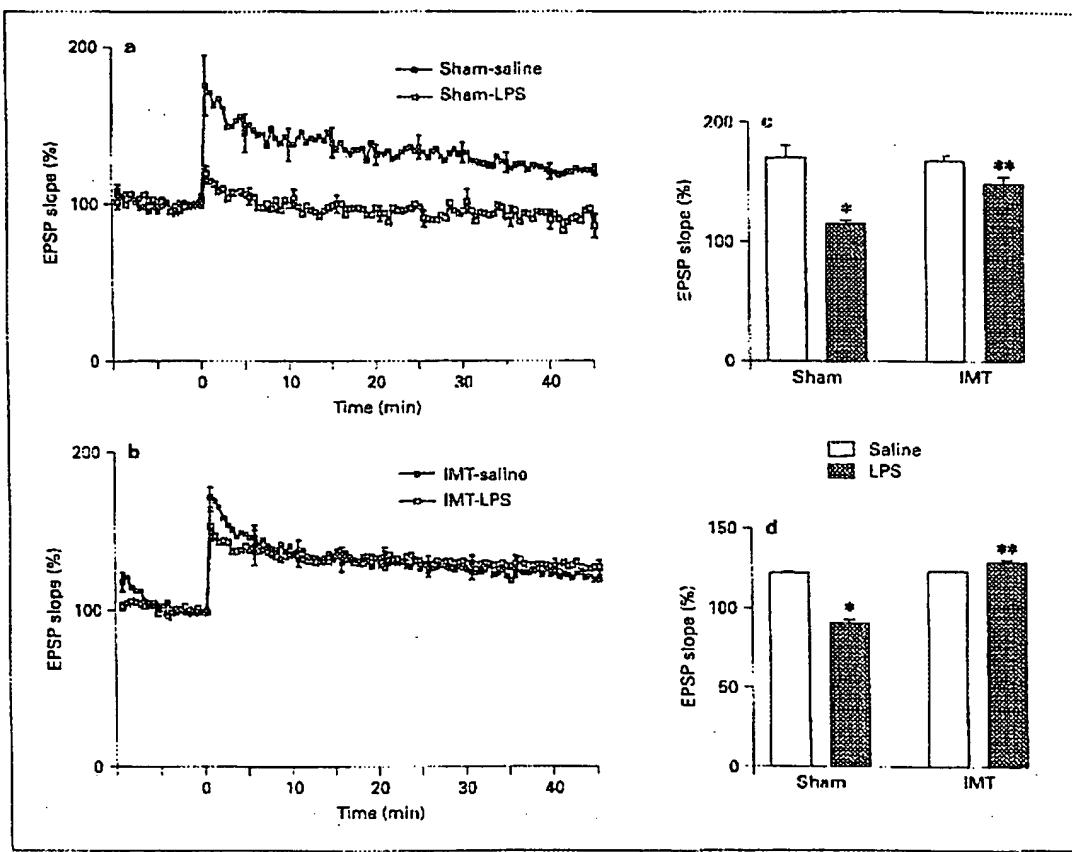


Fig. 1. Intraperitoneal injection of LPS inhibits ITP in perforant path-granule cell synapses (**a**). This inhibitory effect is prevented by pre-treatment with Vasogen's IMT, which exerted no significant effect in saline-treated rats (**b**). The data presented are means of 7–8 observations in each treatment group. Data are expressed as mean percentage change in EPSP slope every 30 s, normalised with respect to the mean value in the 5 min immediately prior to tetanic stimulation. SEM are included for every 10th response. Analysis of the mean values in the 2 min immediately following tetanic stimulation (**c**) and in the last 5 min of the experiment (**d**) indicate that population EPSP slope was significantly decreased in the sham-LPS group (* $p < 0.01$ vs. sham-saline; ANOVA), but that Vasogen's IMT significantly reversed this effect (** $p < 0.01$ vs. sham-LPS; ANOVA). These values are means \pm SEM of 7–8 observations in each case.

equalise for protein concentration. These samples (10 μ l, 1 mg/ml) were added to 10 μ l sample buffer (Tris-HCl 0.5 mM, pH 6.8; glycerol 10%; SDS 10%; β -mercaptoethanol 5%; bromophenol blue 0.05% w/v), boiled for 5 min and loaded onto gels (10% SDS). Proteins were separated by application of a 30-mA constant current for 25–30 min, transferred onto nitrocellulose strips (225 mA for 75 min) and immunoblotted by incubation with an antibody that specifically targets phosphorylated JNK [Santa Cruz Biotechnology, USA; 1:200 in Tris-buffered saline Tween (0.1% Tween-20) containing 1% BSA] for 2 h at room temperature. Nitrocellulose strips were washed and incubated for 2 h at room temperature with secondary antibody (peroxidase-linked anti-mouse IgG; 1:300 dilution; Sigma, UK). Visualization was achieved using SuperSignal West Dura Extended Duration Substrate (Pierce, USA). Immunoblots were immersed in substrate

for 5 min and subsequently exposed to film for 1 s. Film was processed using a Fuji X-ray processor, and quantification of protein bands was achieved by densitometric analysis using two software packages: Grab It (Grab It Annotating Grabber 2.04.7, Synoptics; UVP Ltd., UK) and Gelworks (Gelworks 1D, Version 2.51; UVP Ltd) for photography and densitometry, respectively. Gelworks provides a single value (in arbitrary units), representing the density of each blot; the values presented here are means of data generated from at least 4 separate experiments.

TUNEL Staining

Dissociated cells were prepared by enzymatic and mechanical digestion of fresh hippocampal slices. Slices were incubated with collagenase (0.125%; Sigma) in PBS for 30 min at room temperature,

washed with PBS to terminate collagenase digestion, and then gently triturated with a glass Pasteur pipette before passing through a nylon mesh filter to remove tissue clumps. Cells were then cytospon onto glass microscope slides, fixed with methanol and stored until use.

TUNEL (Terminal deoxynucleotidyl Transferase (TdT)-mediated dUTP Nick-End Labelling) staining, which identifies nuclei with fragmented DNA (a characteristic of apoptotic cells), was performed according to the manufacturer's (Promega, USA) instructions. Briefly, fixed cytospon cells were washed and permeabilised with 0.2% Triton in PBS. Cells were equilibrated in buffer (200 mM potassium cacodylate (pH 6.6 at 25°C), 25 mM Tris-HCl (pH 6.6 at 25°C), 0.2 mM DTT, 0.25 mg/ml BSA, 2.5 mM CoCl₂) for 5 min at room temperature and incubated in TdT reaction mixture (30 µl; 98 µl equilibration buffer, 1 µl biotinylated nucleotide mix, 1 µl TdT enzyme) at 37°C for 1 h. The reaction was terminated by adding 100 µl 2 × SCC (1:10; 2 × SCC:deionised water), endogenous peroxidases were blocked by incubation with H₂O₂ (100 µl; 0.3% in PBS) for 5 min at room temperature, and washed cells were incubated for 30 min at room temperature in streptavidin HRP solution (100 µl; 1:500 in PBS) to allow binding to the biotinylated nucleotides. Diaminobenzidine solution was added to washed cells, and the incubation proceeded for 10 min at room temperature. Cells were washed with deionised water, dehydrated through graded ethanol, cleared with xylene and then slides were mounted in DPX mounting medium and coverslipped. TUNEL-positive cells were expressed as a percentage of the total.

Statistical Analysis

Data were analysed, as appropriate, using either Student's t test for independent means, or a one-way analysis of variance (ANOVA) followed by post hoc Student Newman-Keuls test to determine which conditions were significantly different from each other. Data are expressed as means with standard errors and deemed statistically significant when $p < 0.05$.

Results

In the sham-LPS group, tetanic stimulation delivered to the perforant path 3 h after intraperitoneal injection of LPS resulted in an increase in the mean slope of the population excitatory post-synaptic potential (EPSP) recorded in cell bodies of the granule cells. The mean percentage change (\pm SEM) in the 2 min immediately following tetanic stimulation compared with 5 min immediately before tetanic stimulation was 114.49 ± 2.79 . This was not maintained, however, so that the mean percentage change in population EPSP slope in the last 5 min of the experiment was 90.32 ± 2.42 in the sham-LPS group. The corresponding values in the sham-saline groups of rats were 170.15 ± 10.16 and 121.28 ± 1.20 , respectively (fig. 1a).

The LPS-induced inhibition of LTP was overcome by pre-treatment with Vasogen's IMT. The mean percentage change in population EPSP slope (mean \pm SEM) in the 2 min immediately after tetanic stimulation was 147.44

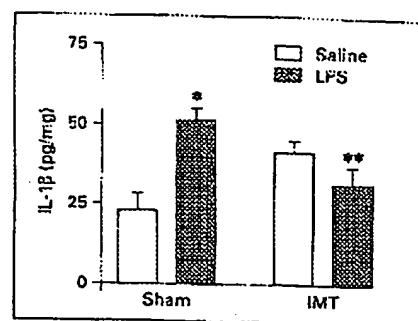


Fig. 2. LPS stimulated a significant increase in the concentration of hippocampal IL-1 β in sham-treated animals (* $p < 0.01$ vs. sham-Saline; ANOVA). This effect was significantly abrogated in animals pre-treated with Vasogen's IMT (** $p < 0.05$ vs. sham-LPS; ANOVA). Data are expressed as means \pm SEM, $n = 7-8$.

\pm 5.84 in the IMT-LPS group compared with 166.85 ± 4.54 in the IMT-saline group. In the last 5 min of the experiment, the values were 128.07 ± 1.46 for the IMT-LPS group and 121.96 ± 0.85 for the IMT-saline group ($n = 7-8$; fig. 1b). The values in the 2 min immediately after tetanic stimulation and in the last 5 min of the experiment were similar in sham-saline and IMT-saline groups ($p > 0.05$). Statistical analysis of the data (fig. 1c, d) revealed that both the early and late phases of LTP were markedly reduced by LPS administration in sham-treated rats ($p < 0.01$ in both cases; ANOVA), and that pre-treatment with Vasogen's IMT significantly attenuated the effect of LPS ($p < 0.01$ in both cases; ANOVA).

Figure 2 shows that IL-1 β concentration in the hippocampus was significantly increased in the sham-LPS group compared to the sham-saline group ($p < 0.01$; ANOVA); this increase was significantly attenuated by pre-treatment with Vasogen's IMT ($p < 0.05$; ANOVA).

The LPS-induced changes in IL-1 β and LTP in sham-LPS rats were associated with an increase in JNK activity in the hippocampus (fig. 3a). In animals treated with Vasogen's IMT, however, these differences were coupled with an attenuated LPS-induced increase in JNK ($p < 0.05$; ANOVA; fig. 3a). Thus JNK activation was increased in the hippocampi of sham-treated animals challenged with LPS [compare lanes 1 (sham-saline) and 2 (sham-LPS)]. This effect was attenuated in rats treated with Vasogen's IMT [compare lanes 2 (sham-LPS) and 4 (IMT-LPS)], which exerted no effect if given on its own [lane 3 (IMT-saline)]. Mean data obtained from densitometric analysis revealed that LPS challenge significantly increased JNK activation in sham-treated animals by

Fig. 3. The LPS-induced increase in JNK activity is blocked by pre-treatment with Vasogen's IMT (a). LPS induces a significant increase in JNK activity in the hippocampi of sham-treated rats, as indicated by an increase in the phosphorylated form of JNK (JNK-1 isoform 46 kD; * p < 0.05 vs. sham-saline; ANOVA). Analysis of the mean data obtained from densitometric analysis indicated that Vasogen's IMT significantly reduced this effect of LPS. (** p < 0.05 vs. sham-LPS; ANOVA). Sample immunoblots indicate the stimulatory effects of LPS (lane 2) on JNK activity in the absence of Vasogen's IMT (compare lanes 1 and 2) and the inhibition of this effect after pre-treatment with Vasogen's IMT (compare lanes 2 and 4). Data are expressed as means \pm SEM, n = 7–8. In vitro, VIP blocks IL-1 β -induced increase in JNK activity (b). IL-1 β induces a significant increase in JNK activity (* p < 0.05 vs. control; ANOVA; compare lanes 1 and 2), but this effect is blocked by co-incubation with VIP (compare lanes 2 and 4). Data are expressed as means \pm SEM, n = 6.

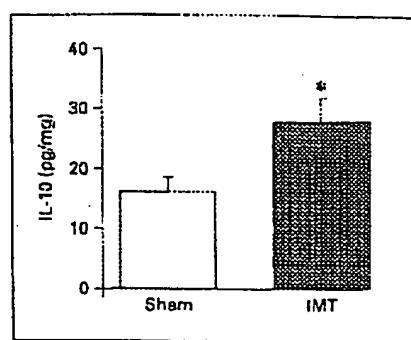
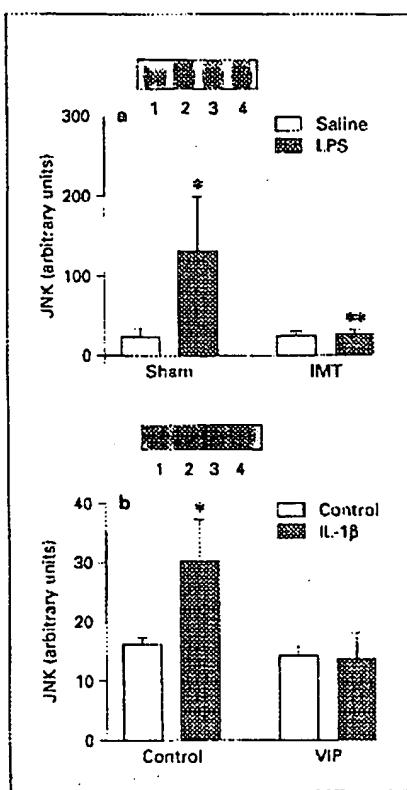


Fig. 4. The concentration of anti-inflammatory cytokine IL-10 was significantly increased in hippocampal tissue as a result of treatment with Vasogen's IMT (* p < 0.05 vs. sham; Student's t test for independent means). Data are expressed as means \pm SEM, n = 7–8.

LPS challenge (p < 0.05; ANOVA), whereas no parallel effect of LPS on JNK activation was observed in tissue prepared from rats treated with Vasogen's IMT.

When JNK activity was analysed after pre-incubating freshly prepared hippocampal synaptosomes from untreated rats in the absence and presence of IL-1 β and the non-specific JNK inhibitor VIP, a VIP-associated attenuation of IL-1 β -induced activity was observed (fig. 3b). In vitro, IL-1 β induced a significant increase in JNK activity (p < 0.05; ANOVA; compare lanes 1 and 2), but this effect was blocked by co-incubation with VIP (compare lanes 2 and 4).

Analysis of IL-10 in the rat hippocampus revealed that Vasogen's IMT was associated with a significant increase in IL-10 relative to sham treatment (p < 0.05; Student's t test for independent means; fig. 4).

Figure 5a demonstrates that the percentage of dissociated cells prepared from fresh hippocampal tissue staining positive for TUNEL was significantly increased in the sham-LPS group compared with the sham-saline group (p < 0.01; ANOVA). Animals treated with Vasogen's IMT did not display this degenerative effect of LPS (p < 0.01;

ANOVA). A representative image of TUNEL-positive cells shows an increased number of apoptotic cells after LPS injection, as evidenced by increased number of cells displaying dark brown stained nuclei, i.e. TUNEL-positive cells (fig. 5bii). This contrasts with cells prepared from hippocampi of sham-saline (fig. 5bi) and IMT-saline rats (fig. 5biii). Figure 5biv shows a reduction in the number of cells displaying TUNEL-positive staining in the IMT-LPS group.

Discussion

The objective of this study was to investigate the possibility that pre-treating rats with Vasogen's IMT may block the LPS-induced inhibitory effects on synaptic plasticity in the hippocampus. Accordingly, the data demonstrate that the LPS-induced inhibition of LTP in perforant path-granule cell synapses was abrogated by pre-treatment with Vasogen's IMT.

Systemic injection of LPS in sham-treated animals induced an increase of IL-1 β in the hippocampus, a find-

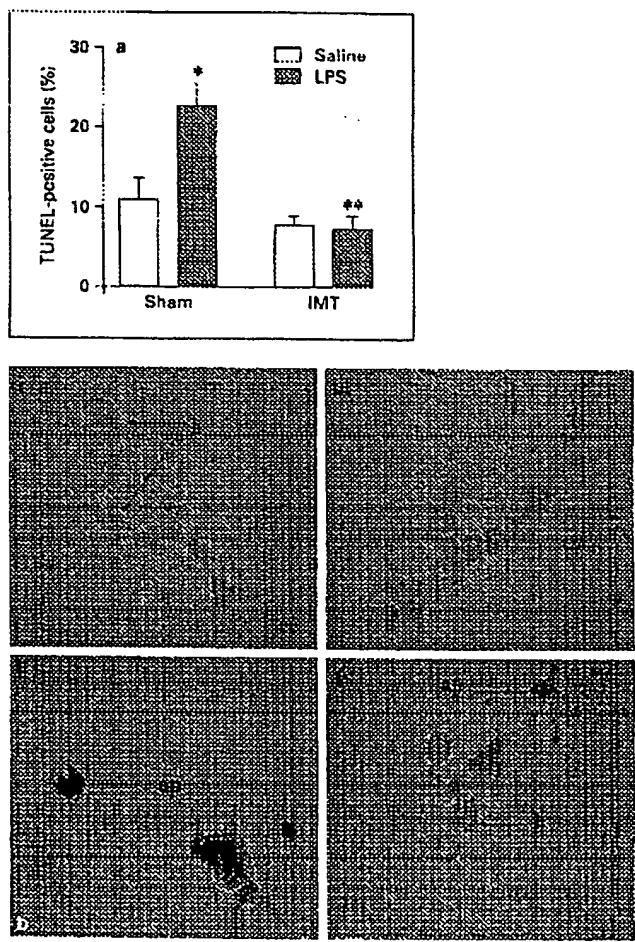


Fig. 5. The LPS-induced apoptotic changes in hippocampal cells were inhibited by pre-treatment with Vasogen's IMT. Cytospun cells were prepared from the hippocampi of rats and the mean data, obtained by counting 200 cells on each coverslip, show a significant increase in the percentage of apoptotic cells in the sham-LPS group compared with sham-saline rats (* $p < 0.01$ vs. sham-saline; ANOVA). This percentage increase is reversed by pre-treatment with Vasogen's IMT (** $p < 0.01$ vs. sham-LPS; ANOVA; a). Data are expressed as mean \pm SEM, $n = 5$. Representative image TUNEL staining displays healthy (b) and apoptotic (ap) cells (b). There is an increased number of dark brown stained cells prepared from hippocampi of rats injected with LPS (ii) compared with cells prepared from saline-injected control rats (i), and rats treated with IMT only (iii). Pre-treatment with IMT reversed the effects of LPS with fewer cells displaying brown staining (iv). Scale bar is 20 μ m.

ing that supports earlier reports [2, 17]. It has been proposed that LPS may inhibit LTP in perforant path-granule cell synapses as a consequence of an LPS-induced increase in IL-1 β concentration in the hippocampus [2]. Indeed, it has previously been reported that intracerebroventricular injection of IL-1 β inhibits LTP in perforant path-granule cell synapses in vivo [3, 8], and that IL-1 β attenuates LTP in dentate gyrus in vitro [18]. Pre-treatment with Vasogen's IMT prevented the LPS-stimulated increase in hippocampal IL-1 β concentration, as well as the LPS-induced inhibition of LTP.

Data from this laboratory have demonstrated a stimulatory effect of both LPS and IL-1 β on JNK activation [2, 19]. As a consequence of the preventative effect of Vasogen's IMT on the LPS-induced increase in IL-1 β concentration, the stimulatory effect of LPS on JNK activity was also attenuated. Here we also report that co-incubation of synaptosomes in the presence of IL-1 β and the non-specific JNK inhibitor VIP blocks the IL-1 β -induced increased activation of JNK. Thus it seems reasonable to propose that Vasogen's IMT may exert its protective effect on synaptic function by acting to prevent this LPS-induced signalling event.

Another consequence of peripheral administration of LPS is neuronal degeneration, as demonstrated by increased numbers of cells whose nuclei display fragmented DNA, a characteristic associated with apoptosis [2, 6, 20]. Accordingly, we have demonstrated a significant increase in the percentage of TUNEL-positive cells in the hippocampus as a result of LPS administration in sham-treated animals. It is possible that the impairment in LTP due to LPS injection in sham-treated animals may be due to degenerative changes in hippocampal cells. Thus it is not unreasonable to suggest that the prevention of LPS-induced inhibition of LTP by Vasogen's IMT is paralleled by a prevention of LPS-induced cell death. The present evidence, which shows an abrogation of LPS-induced increase in TUNEL-positive hippocampal cells by Vasogen's IMT, supports this proposal. Concurrent with this finding is the observation that pre-treatment with Vasogen's IMT reduces apoptosis after acute renal ischemia/reperfusion injury in dogs, as estimated by the reduction in mitochondrial membrane potential [21].

It has recently been shown that intracerebroventricular administration of the anti-inflammatory cytokine IL-10 in vivo reverses IL-1 β -induced impairment of LTP and JNK activation in the hippocampus [8]. Injection of IL-10 has also been shown to reduce LPS-induced fever [22] and the behavioural effects induced by LPS [23]. In addition, IL-10 has been shown to confer protection against oligo-

dcndroglial death evoked by LPS/IFN- γ in vitro [24]. In the present study, pre-treatment with Vasogen's IMT caused a significant increase in IL-10 concentration in the hippocampus. This finding is consistent with the observation that administration of Vasogen's IMT inhibits Th1-mediated contact hypersensitivity in mice [11], and the level of suppression was comparable to that seen with animal models using IL-10 [25]. Therefore, it is possible that in the present experimental paradigm, Vasogen's IMT may exert its beneficial effects by suppressing the Th1 response with a concomitant release of anti-inflammatory IL-10, as suggested by the obstruction of LPS-induced pro-inflammatory effects. Whether the IMT-induced central decrease in IL-1 β and the increase in IL-10 observed in the study are due to a peripheral decrease in IL-1 β and increase in IL-10 is as yet unknown. Current information

on the mechanism of immune molecule trafficking across the blood-brain barrier is limited. However, some signals that regulate immune cell traffic include intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on activated brain endothelia, and their counter-receptors LFA-1 and VLA-4 on immune cells. It remains to be investigated whether peripheral administration of Vasogen's IMT induces its central effects by activation of these mediators.

Although the exact mechanism of action of Vasogen's IMT remains to be elucidated, there is clear evidence from the data presented in this study that pre-treatment with the therapy confers a protective effect on the organism by preventing LPS-induced impairment of synaptic function and the resultant detrimental effects in the hippocampus of the rat.

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